



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : A61K 48/00, 38/00, C12P 21/00, C07K 5/00	A1	(11) International Publication Number: WO 00/07631 (43) International Publication Date: 17 February 2000 (17.02.00)
(21) International Application Number: PCT/US99/17596 (22) International Filing Date: 4 August 1999 (04.08.99) (30) Priority Data: 60/095,267 4 August 1998 (04.08.98) US (71) Applicant (for all designated States except US): THE HENRY M. JACKSON FOUNDATION [US/US]; Suite 600, 1401 Rockville Pike, Rockville, MD 20852 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): QUINNAN, Gerald, V., Jr. [US/US]; Uniformed Services, University of the Health Sciences (Department of Preventive Medicine and Biometrics), 4301 Jones Bridge Road, Bethesda, MD 20814-4799 (US). ZHANG, Peng, Fei [CN/US]; Department of Preventive Medicine and Biometrics, 4301 Jones Bridge Road, Bethesda, MD 20814-4799 (US). (74) Agent: ADLER, Reid, G.; Morgan, Lewis & Bockius LLP, 1800 M Street, N.W., Washington, DC 20036-5869 (US).		(81) Designated States: AU, CA, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i>
(54) Title: EXPRESSION AND CHARACTERIZATION OF HIV-1 ENVELOPE PROTEIN ASSOCIATED WITH A BROADLY REACTIVE NEUTRALIZING ANTIBODY RESPONSE (57) Abstract <p>The present invention relates to HIV-1 envelope proteins from a donor with non-progressive HIV-1 infection whose serum contains broadly cross-reactive, primary virus neutralizing antibody. The invention also relates to isolated or purified proteins and protein fragments that share certain amino acids at particular positions with the foregoing HIV-1 proteins.</p>		

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**Expression and Characterization of HIV-1 Envelope Protein Associated with a
Broadly Reactive Neutralizing Antibody Response**

5 **Inventors: Gerald V. Quinnan, Jr. & Peng Fei Zhang**

Related Applications

This application is related to U.S. Provisional Application 60/095,267 filed
10 August 4, 1998, which is herein incorporated by reference in its entirety.

Technical Field

The present invention relates to HIV-1 envelope proteins and peptides derived
15 from the donor of the Neutralizing Reference Human Serum (2) which is noted for its
capacity to neutralize primary HIV isolates of varied subtypes.

Acknowledgment of Federal Support

20 The present invention arose in part from research funded by the following federal
grant monies: NIH AI37436 and AI44339, and USUHS R087E2

Background of the Invention

25 The development of a successful vaccine against HIV infection or a vaccine agent
capable of preventing HIV disease progression has been a public health goal for over 15
years. One of the immune responses that may be required to elicit a protective immune
response against HIV infection is the generation of antibodies that are virus neutralizing.

The target of HIV-1 neutralizing antibodies (NA) is the envelope glycoprotein
30 complex. This complex is a multimeric structure composed of three or four copies each
of the gp120 surface and gp41 transmembrane glycoproteins (Luciw, 1996). There are a

number of neutralization domains on each of the three or four heterodimeric components of the complex (Thali *et al.*, 1992, 1993; Zwart *et al.*, 1991; Moore *et al.*, 1993; Trkola *et al.*, 1996; Muster *et al.*, 1993; Cotropia *et al.*, 1996; Sabri *et al.*, 1996). The amino acid compositions of the proteins vary substantially from strain to strain. Some of the
5 neutralization domains are in regions which tend to vary greatly, while others are in regions which tend to be highly conserved. The variable neutralization domains include those in variable (V) regions 1, 2, and 3 of gp120, while the conserved domains include the primary receptor binding site, and other epitopes in gp120 and gp41. Amino acid sequence variation is undoubtedly the explanation for the variation that is seen in
10 specificity of neutralization sensitivity among virus strains. However, it has not been possible to classify antigenic subtypes of HIV-1 based on genetic analyses, and various regions of the envelope complex even outside of the neutralization domains have been shown to contribute to antigenic variability (Thali *et al.*, 1994; Back *et al.*, 1993).

Recent findings indicate that the neutralization of primary isolates of HIV may be
15 mediated primarily by antibodies directed against non-V3 region epitopes expressed on the oligomeric complex but not on monomeric gp120, while laboratory adapted strains are more readily neutralized by antibodies directed against V3 (Hioe *et al.*, 1997; VanCott *et al.*, 1997). The identity of the non-V3 epitopes recognized on primary isolates is not established. The presence of antibodies which have broadly neutralizing activity against
20 primary isolates of many subtypes of HIV-1 in sera from infected people is unusual, but the nature of the envelope proteins in individuals with such antibodies may be of interest for defining the epitopes which may be broadly immunogenic in vaccines.

25 Summary of the Invention

The present inventors have cloned and characterized the envelope genes from the donor of human serum which is noted for its capacity to neutralize primary HIV isolates of various subtype (Vujcic, *et al.* 1995, D'Souza *et al.*, 1991).

The invention includes an isolated HIV envelope protein or fragment thereof
30 which, when injected into a mammal, induces the production of broadly cross-reactive neutralizing anti-serum against multiple strains of HIV-1.

The invention further includes an isolated HIV envelope protein or fragment thereof comprising a proline at a position corresponding to amino acid residue 313, a methionine at a position corresponding to amino acid residue 314 and a glutamine at a position corresponding to amino acid residue 325 of SEQ ID NO:1.

5 In another embodiment, the invention includes an isolated HIV envelope glycoprotein or fragment thereof comprising an alanine at a position corresponding to amino acid residue 167 of SEQ ID NO:1.

The invention also includes an isolated HIV envelope protein comprising the amino acid sequence of SEQ ID NO:1 as well as an isolated nucleic acid molecule
10 encoding the envelope protein.

Compositions for eliciting an immune response, such as vaccines, immunogenic compositions and attenuated viral vaccine delivery vectors comprising the envelope proteins, peptides and nucleic acids encoding such proteins and peptides of the invention are also included. Methods for generating antibodies in a mammal comprising
15 administering one or more of these proteins, peptides and nucleic acids, in an amount sufficient to induce the production of the antibodies, is also included in the invention.

The invention also comprises a diagnostic reagent comprising one or more of the isolated HIV-1 envelope proteins and methods for detecting broadly cross-reactive neutralizing anti-serum against multiple strains of HIV-1.

20

Brief Description of the Drawings

Figure 1: Phylogenetic analysis of the gp120 and gp41 nucleotide coding sequences of clone R2. Alignments were performed using the Clustal algorithm of
25 Higgins and Sharp in the program DNA Star (Higgins *et al.*, 1989; Saitou *et al.*, 1987; Myers *et al.*, 1988). The graphs at the bottom of the two figures indicate the percent similarity distances represented by the dendrograms. Gene bank accession numbers for the sequences represented are: MW 959, U08453; MW960, U08454; D747, X65638; BR020, U27401; BR029, U27413; RU131, U30312; UG975, U27426; AD8, M60472;
30 HXB, K03455; NDK, M27323; Z2Z6, M22639; UG021, U27399; CM235, L03698;

TH022, U09139; TH006, U08810; UG275, L22951; SF1703, M66533; RW020, U08794; RW009, U08793; U455, M62320; and Z321, M15896.

Figure 2: Neutralization of clade B viruses and pseudoviruses by sera from 10 male residents of the Baltimore/Washington, D.C. area collected from 1985-1990 in the Multicenter AIDS Cohort Study. The P9 and P10 viruses (P9-V and P10-V) are primary isolates from two of the serum donors (Quinnan *et al.*, 1998). The neutralization assays were performed in PM1 cells, as described in the Examples. Each point represents the results obtained with an individual serum. The open bars represent the standard deviations about the geometric means, indicated by the midlines. The numbers above the results obtained using pseudoviruses indicate the probabilities obtained from testing the null hypothesis by paired t testing comparing the individual pseudoviruses to R2.

Figure 3 (A): Inhibition of Reference 2-mediated neutralization of pseudoviruses by synthetic V3 peptides. The neutralization endpoints for 90% neutralization were calculated as described previously (Quinnan *et al.*, 1999; Quinnan *et al.*, 1998; Zhang *et al.*, 1999; Park *et al.*, 1998). Results shown are means of triplicate determinations. Dose-response effects of R2 linear 17-mer (open square) and cyclic (closed square) and the 93TH966.8 cyclic (shaded square) V3 peptides on neutralization of clone R2 pseudovirus. The peptide concentrations are 3×10 raised to the indicated power.

Figure 3 (B): Comparative inhibitory effects of peptides on neutralization of R2 and MN (clone V5) pseudoviruses. All peptides were tested at $15 \mu\text{g/ml}$. The linear peptides (L) corresponded to the apical sequences of the respective V3 loops. The cyclic peptides (C) corresponded to the full lengths of the respective V3 regions of the different strains. Neutralization in the absence of peptide (None), is also shown.

Figure 4 (A): Effect of cyclic R2 V3 peptide on neutralization of pseudoviruses. Fold inhibition of neutralization was calculated as the ratio of the 50% neutralization titer obtained in the absence of peptide compared to that obtained in the presence of cyclic R2 V3 peptide ($15 \mu\text{g/ml}$). All assays were performed in triplicate. Neutralization titers

were calculated at the midpoints of the infectivity inhibition curves, since the curves tended to be most parallel in this region. Similar results were obtained comparing 90% neutralization endpoints. Peptide inhibition of neutralization of R2 pseudovirus by sera from MACS donors (donor numbers 1-10), two assays each, and by Reference 2. Results are shown for two determinations for each serum from the MACS donors and for 12 assays of Reference 2 performed during the same time intervals as the other experiments shown in panels (A) and (B).

Figure 4 (B): Peptide inhibition of neutralization of pseudoviruses expressing MACS patient envelopes (patient numbers 3, 4, 6, 8, 9, and 10) by Reference 2. Results of two or three separate assays of each pseudovirus are shown.

Modes of Carrying Out the Invention

General Description

A goal of immunization against HIV is to induce neutralizing antibody (NA) responses broadly reactive against diverse strains of virus. The present inventors have studied envelope protein from a donor with non-progressive HIV-1 infection whose serum contains broadly cross-reactive, primary virus NA. DNA was extracted from lymphocytes, which had been collected approximately six and twelve months prior to the time of collection of the cross reactive serum, *env* genes were synthesized by nested PCR, cloned, expressed on pseudoviruses, and phenotyped in NA assays. Two clones from each time point had identical V3 region nucleotide sequences, utilized CCR5 but not CXCR4 for cell entry, and had similar reactivities with two reference sera. Analysis of the full nucleotide sequence of one clone demonstrated it to be subtype B, with a predicted GPGRF apical V3 sequence, normal predicted glycosylation, and an intact reading frame. Infectivity assays of R2 pseudovirus in HOS cells expressing CD4 and various coreceptors demonstrated the envelope to be CCR5 dependent. R2 pseudovirus was compared to others expressing *env* genes of various clades for neutralization by sera from donors in the United States (presumed or known subtype B infections), and from individuals infected with subtypes A, C, and E viruses. Neutralization by the sera from

donors in the United States of pseudoviruses expressing R2 and other clade B envs was similarly low to moderate, although R2 was uniquely neutralized by all. R2 was neutralized by sera from people infected with clades A-F, while other clade B, D, E and G pseudoviruses were neutralized less often. One highly sensitive clade C pseudovirus was neutralized by all the sera, although the titers varied more than 250-fold. The results suggest that the epitope(s) which induced the cross-clade reactive NA in Donor 2 may be expressed on the R2 envelope.

The present invention relates to HIV-1 envelope proteins from this donor who had non-progressive HIV-1 infection whose serum contains broadly cross-reactive, primary virus neutralizing antibody. The invention also relates to isolated or purified proteins and protein fragments that share certain amino acids at particular positions with the foregoing HIV-1 proteins.

Specific Embodiments

Proteins and Peptides

Proteins and peptides of the invention include the full length envelope protein having the amino acid sequence of Table 3 (SEQ ID NO:1), gp120 having the amino acid sequence corresponding to gp120 in Table 3 (amino acids: 1-520 of SEQ ID NO:1), gp41 having the amino acid sequence corresponding to gp41 in Table 3 (amino acids 521-866 of SEQ ID NO:1), as well as polypeptides and peptides corresponding to the V3 domain and other domains such as V1/V2, C3, V4, C4 and V5. These domains correspond to the following amino acid residues of SEQ ID NO:1:

DOMAIN	AMINO ACID RESIDUES
C1	30-124
V1	125-162
V2	163-201
C2	202-300
V3	301-336
C3	337-387

DOMAIN	AMINO ACID RESIDUES
V4	388-424
C4	425-465
V5	466-509
C5	510-520

5

Polypeptides and peptides comprising any single domain may be of variable length but include the amino acid residues of Table 3 (SEQ ID NO:1) which differ from previously sequenced envelope proteins. For instance, peptides of the invention which include all or part of the V3 domain may comprise the sequence: PM X₁ X₂ X₃ X₄ X₅ X₆ X₇ X₈ X₉ X₁₀ Q, wherein X₁-X₁₀ are any natural or non-natural amino acids (P refers to Proline, M refers to methionine and Q refers to Glutamine). Non-natural amino acids include, for example, beta-alanine (beta-Ala), or other omega-amino acids, such as 3-amino propionic, 2,3-diamino propionic (2,3-diaP), 4-amino butyric and so forth, alpha-aminisobutyric acid (Aib), sarcosine (Sat), ornithine (Orn), citrulline (Cit), t-butylalanine (t-BuA), t-butylglycine (t-BuG), N-methylisoleucine (N-MeIle), phenylglycine (Phg), and cyclohexylalanine (Cha), norleucine (Nle), cysteic acid (Cya) 2-naphthylalanine (2-Nal); 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (Tic); beta-2-thienylalanine (Thi); and methionine sulfoxide (MSO). Preferably, peptides of the invention are 60%, 70%, 80% or more preferably, 90% identical to the V3 region of the HIV envelope protein of Table 3 (SEQ ID NO:1). Accordingly, V3 peptides of the invention comprise about 13 amino acids but may be 14, 15, 17, 20, 25, 30, 35, 36, 39, 40, 45, 50 or more amino acids in length. In one embodiment, a V3 peptide of 13 amino acids in length consists of the sequence PMGPGRAFYTGTGQ (amino acids 313-325 of Table 3 (SEQ ID NO:1)).

25

In another embodiment of the invention, polypeptides and peptides comprising all or part of the V1/V2 domain comprise an amino acid sequence with an alanine residue at a position corresponding to amino acid 167 Table 3 (SEQ ID NO:1). For instance, peptides of the invention spanning the V1/V2 domain may comprise the sequence FNIATSIG (residues 164-171 of SEQ ID NO:1) and may be about 8, 9, 10, 15, 20, 25,

30, 35, 40, 45, 50 or more amino acids in length. As used herein, "at a position corresponding to" refers to amino acid positions in HIV envelope proteins or peptides of the invention which are equivalent to a given amino acid residue in the sequence of Table 1 (SEQ ID NO:1) in the context of the surrounding residues.

5 The peptides of the present invention may be prepared by any known techniques. Conveniently, the peptides may be prepared using the solid-phase synthetic technique initially described by Merrifield (1965), which is incorporated herein by reference. Other peptide synthesis techniques may be found, for example, in Bodanszky *et al.*, *Peptide Synthesis*, 2d ed. (New York, Wiley, 1976).

10

Nucleic acids and Recombinant Expression of Peptide or Proteins

 Proteins and peptides of the invention may be prepared by any available means, including recombinant expression of the desired protein or peptide in eukaryotic or prokaryotic host cells (see U.S. Patent 5,696,238). Methods for producing proteins or peptides of the invention for purification may employ conventional molecular biology, microbiology, and recombinant DNA techniques within the ordinary skill level of the art. Such techniques are explained fully in the literature. See, for example, Maniatis *et al.*, *Molecular Cloning: A Laboratory Manual*, 2d ed. (Cold Spring Harbor, Cold Spring Harbor Laboratory Press, 1989); Glover, *DNA Cloning: A Practical Approach*, Vols. 1-4
15 (Oxford, IRL Press, 1985); Gait, *Oligonucleotide Synthesis: A Practical Approach* (Oxford, IRL Press, 1984); Hames & Higgins, *Nucleic Acid Hybridisation: A Practical Approach* (Oxford, IRL Press, 1985); Freshney, *Animal Cell Culture: A Practical Approach* (Oxford, IRL Press, 1992); Perbal, *A Practical Guide To Molecular Cloning* (New York, Wiley, 1984).
20

25 The present invention further provides nucleic acid molecules that encode the proteins or peptides of the invention. Such nucleic acid molecules can be in an isolated form, or can be operably linked to expression control elements or vector sequences. The present invention further provides host cells that contain the vectors via transformation, transfection, electroporation or any other art recognized means of introducing a nucleic
30 acid into a cell.

As used herein, a "cell line" is a clone of a primary cell that is capable of stable growth *in vitro* for many generations.

A DNA "coding sequence" is a double-stranded DNA sequence which is transcribed and translated into a polypeptide *in vivo* when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxy) terminus. A polyadenylation signal and transcription termination sequence will usually be located 3' to the coding sequence.

A "heterologous" region of the DNA construct is an identifiable segment of DNA within a larger DNA molecule that is not found in association with the larger molecule in nature. Thus, when the heterologous region encodes a mammalian gene, the gene will usually be flanked by DNA that does not flank the mammalian genomic DNA in the genome of the source organism. Another example of a heterologous coding sequence is a construct where the coding sequence itself is not found in nature (*e.g.*, a cDNA where the genomic coding sequence contains introns, or synthetic sequences having codons different than the native gene). Allelic variations or naturally-occurring mutational events do not give rise to a heterologous region of DNA as defined herein.

As used herein, "naked DNA" means nucleic acid molecules that are free from viral particles, particularly retroviral particles. This term also means nucleic acid molecules which are free from facilitator agents including but not limited to the group comprising: lipids, liposomes, extracellular matrix-active enzymes, saponins, lectins, estrogenic compounds and steroidal hormones, hydroxylated lower alkyls, dimethyl sulfoxide (DMSO) and urea.

As used herein, a "nucleic acid molecule" refers to the polymeric form of deoxyribonucleotides (adenine, guanine, thymine, and/or cytosine) in either its single stranded form, or in double-stranded helix as well as RNA. This term refers only to the primary and secondary structure of the molecule and is not limited to any particular tertiary form. In discussing the structure of particular double-stranded DNA molecules, sequences may be described herein according to the normal convention of giving only the sequence in the 5' to 3' direction along the nontranscribed strand of DNA (*e.g.*, the strand having a sequence homologous to the mRNA). Transcriptional and translational control

sequences are DNA regulatory sequences, such as promoters, enhancers, polyadenylation signals, terminators, and the like, that provide for the expression of a coding sequence in a host cell.

As used herein, a "promoter sequence" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. For purposes of defining the present invention, the promoter sequence is bounded (inclusively) at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence will be found a transcription initiation site, as well as protein binding domains responsible for the binding of RNA polymerase. Eukaryotic promoters will often, but not always, contain "TATA" boxes and "CAT" boxes.

As used herein, a "replicon" is any genetic element (*e.g.*, plasmid, chromosome, virus) that functions as an autonomous unit of DNA replication *in vivo*; *i.e.*, capable of replication under its own control.

A "signal sequence" can be included before the coding sequence or the native amino acid signal sequence from the envelope protein of Table 3 may be used. This sequence encodes a signal peptide, N-terminal to the polypeptide, that communicates to the host cell to direct the polypeptide to the cell surface or secrete the polypeptide into the media. This signal peptide is clipped off by the host cell before the protein leaves the cell. Signal sequences can be found associated with a variety of proteins native to prokaryotes and eukaryotes. For instance, alpha-factor, a native yeast protein, is secreted from yeast, and its signal sequence can be attached to heterologous proteins to be secreted into the media (See U.S. Pat. No. 4,546,082, and EP 0116201). Further, the alpha-factor and its analogs have been found to secrete heterologous proteins from a variety of yeast, such as *Saccharomyces* and *Kluyveromyces*, (EP 88312306.9; EP 0324274 publication, and EP 0301669). An example for use in mammalian cells is the tPA signal used for expressing Factor VIIIc light chain.

As used herein, DNA sequences are "substantially homologous" when at least about 85% (preferably at least about 90% and most preferably at least about 95%) of the nucleotides match over the defined length of the DNA sequences. Sequences that are

substantially homologous can be identified in a Southern hybridization experiment under, for example, stringent conditions as defined for that particular system. Defining appropriate hybridization conditions is within the skill of the art. See, for example, Maniatis *et al.*, *supra*.

5 A cell has been "transformed" by exogenous or heterologous DNA when such DNA as been introduced inside the cell. The transforming DNA may or may not be integrated (covalently linked) into chromosomal DNA making up the genome of the cell. In prokaryotes, for example, the transforming DNA may be maintained on an episomal element such as a plasmid or viral vector. With respect to eukaryotic cells, a stably
10 transformed cell is one in which the transforming DNA has become integrated into a chromosome so that it is inherited by daughter cells through chromosome replication. This stability is demonstrated by the ability of the eukaryotic cell to establish cell lines or clones comprised of a population of daughter cells containing the transforming DNA.

 A coding sequence is "under the control" of transcriptional and translational
15 control sequences in a cell when RNA polymerase transcribes the coding sequence into mRNA, which is then translated into the protein encoded by the coding sequence.

 As used herein, a "vector" is a replicon, such as plasmid, phage or cosmid, to which another DNA segment may be attached so as to bring about the replication of the attached segment.

20 Vectors are used to simplify manipulation of the DNA which encodes the HIV proteins or peptides, either for preparation of large quantities of DNA for further processing (cloning vectors) or for expression of the HIV proteins or peptides (expression vectors). Vectors comprise plasmids, viruses (including phage), and integrated DNA fragments, *i.e.*, fragments that are integrated into the host genome by recombination.
25 Cloning vectors need not contain expression control sequences. However, control sequences in an expression vector include transcriptional and translational control sequences such as a transcriptional promoter, a sequence encoding suitable ribosome binding sites, and sequences which control termination of transcription and translation. The expression vector should preferably include a selection gene to facilitate the stable
30 expression of HIV gene and/or to identify transformants. However, the selection gene for

maintaining expression can be supplied by a separate vector in cotransformation systems using eukaryotic host cells.

Suitable vectors generally will contain replicon (origins of replication, for use in non-integrative vectors) and control sequences which are derived from species compatible with the intended expression host. By the term "replicable" vector as used herein, it is intended to encompass vectors containing such replicons as well as vectors which are replicated by integration into the host genome. Transformed host cells are cells which have been transformed or transfected with vectors containing HIV peptide or protein encoding DNA. The expressed HIV proteins or peptides may be secreted into the culture supernatant, under the control of suitable processing signals in the expressed peptide, *e.g.* homologous or heterologous signal sequences.

Expression vectors for host cells ordinarily include an origin of replication, a promoter located upstream from the HIV protein or peptide coding sequence, together with a ribosome binding site, a polyadenylation site, and a transcriptional termination sequence. Those of ordinary skill will appreciate that certain of these sequences are not required for expression in certain hosts. An expression vector for use with microbes need only contain an origin of replication recognized by the host, a promoter which will function in the host, and a selection gene.

Commonly used promoters are derived from polyoma, bovine papilloma virus, CMV (cytomegalovirus, either murine or human), Rouse sarcoma virus, adenovirus, and simian virus 40 (SV40). Other control sequences (*e.g.*, terminator, polyA, enhancer, or amplification sequences) can also be used.

An expression vector is constructed so that the HIV protein or peptide coding sequence is located in the vector with the appropriate regulatory sequences, the positioning and orientation of the coding sequence with respect to the control sequences being such that the coding sequence is transcribed and translated under the "control" of the control sequences (*i.e.*, RNA polymerase which binds to the DNA molecule at the control sequences transcribes the coding sequence). The control sequences may be ligated to the coding sequence prior to insertion into a vector, such as the cloning vectors described above. Alternatively, the coding sequence can be cloned directly into an expression vector which already contains the control sequences and an appropriate

restriction site. If the selected host cell is a mammalian cell, the control sequences can be heterologous or homologous to the HIV coding sequence, and the coding sequence can either be genomic DNA containing introns or cDNA.

Higher eukaryotic cell cultures may be used to express the proteins of the present invention, whether from vertebrate or invertebrate cells, including insects, and the procedures of propagation thereof are known. See, for example, Kruse & Patterson, *Tissue Culture* (New York, Academic Press, 1973).

Suitable host cells for expressing HIV proteins or peptides in higher eukaryotes include: monkey kidney CVI line transformed by SV40 (COS-7, ATCC CRL1651); baby hamster kidney cells (BHK, ATCC CRL10); Chinese hamster ovary-cells-DHFR (Urlaub & Chasin, 1980); mouse Sertoli cells (Mather, 1980); monkey kidney cells (CVI ATCC CCL70); African green monkey kidney cells (VERO76, ATCC CRL1587); human cervical carcinoma cells (HeLa, ATCC CCL2); canine kidney cells (MDCK, ATCC CCL34); buffalo rat liver cells (BRL3A, ATCC CRL1442); human lung cells (W138, ATCC CCL75); human liver cells (HepG2, HB8065); mouse mammary tumor (MMT 060652, ATCC CCL51); rat hepatoma cells (Baumann *et al.*, 1980) and TRI cells (Mather *et al.*, 1982).

It will be appreciated that when expressed in mammalian tissue, the recombinant HIV gene products may have higher molecular weights than expected due to glycosylation. It is therefore intended that partially or completely glycosylated forms of HIV preproteins or peptides having molecular weights somewhat different from 160, 120 or 41 kD are within the scope of this invention.

Other preferred expression vectors are those for use in eukaryotic systems. An exemplary eukaryotic expression system is that employing vaccinia virus, which is well-known in the art. See, for example, Macket *et al.* (1984); Glover, *supra*; and WO 86/07593. Yeast expression vectors are known in the art. See, for example, U.S. Patents 4,446,235; 4,443,539; 4,430,428; and EP 103409; EP 100561; EP 96491.

Another preferred expression system is vector pHSI, which transforms Chinese hamster ovary cells (see WO 87/02062). Mammalian tissue may be cotransformed with DNA encoding a selectable marker such as dihydrofolate reductase (DHFR) or thymidine kinase and DNA encoding the HIV protein or peptide. If wild type DHFR gene is

employed, it is preferable to select a host cell which is deficient in DHFR, thus permitting the use of the DHFR coding sequence as marker for successful transfection in hgt medium, which lacks hypoxanthine, glycine, and thymidine. An appropriate host cell in this case is the Chinese hamster ovary (CHO) cell line deficient in DHFR activity,
5 prepared and propagated as described by Urlaub & Chasin, (1980).

Depending on the expression system and host selected, HIV proteins or peptides are produced by growing host cells transformed by an exogenous or heterologous DNA construct, such as an expression vector described above under conditions whereby the HIV protein is expressed. The HIV protein or peptide is then isolated from the host cells
10 and purified. If the expression system secretes the protein or peptide into the growth media, the protein can be purified directly from cell-free media. The selection of the appropriate growth conditions and initial crude recovery methods are within the skill of the art.

Once a coding sequence for an HIV protein or peptide of the invention has been
15 prepared or isolated, it can be cloned into any suitable vector and thereby maintained in a composition of cells which is substantially free of cells that do not contain an HIV coding sequence. Numerous cloning vectors are known to those of skill in the art. Examples of recombinant DNA vectors for cloning and host cells which they can transform include the various bacteriophage lambda vectors (*E. coli*), pBR322 (*E. coli*), pACYC177 (*E. coli*),
20 pKT230 (gram-negative bacteria), pGV1106 (gram-negative bacteria), pLAFRI (gram-negative bacteria), pME290 (*non-E. coli* gram-negative bacteria), pHV14 (*E. coli* and *Bacillus subtilis*), pBD9 (*Bacillus*), pIJ61 (*Streptomyces*). pUC6 (*Streptomyces*), actinophage, fC31 (*Streptomyces*). YIpS (*Saccharomyces*), YCp19 (*Saccharomyces*), and bovine papilloma virus (mammalian cells). See generally, Glover, *supra*; T. Maniatis *et al.*, *supra*; and Perbal, *supra*.
25

Fusion Proteins

HIV envelope fusion proteins and methods for making such proteins have been previously described (U.S. Patent 5,885,580). It is now a relatively straight forward
30 technology to prepare cells expressing a foreign gene. Such cells act as hosts and may include, for the fusion proteins of the present invention, yeasts, fungi, insect cells, plants

cells or animals cells. Expression vectors for many of these host cells have been isolated and characterized, and are used as starting materials in the construction, through conventional recombinant DNA techniques, of vectors having a foreign DNA insert of interest. Any DNA is foreign if it does not naturally derive from the host cells used to
5 express the DNA insert. The foreign DNA insert may be expressed on extrachromosomal plasmids or after integration in whole or in part in the host cell chromosome(s), or may actually exist in the host cell as a combination of more than one molecular form. The choice of host cell and expression vector for the expression of a desired foreign DNA largely depends on availability of the host cell and how fastidious it is, whether the host
10 cell will support the replication of the expression vector, and other factors readily appreciated by those of ordinary skill in the art.

The foreign DNA insert of interest comprises any DNA sequence coding for fusion proteins including any synthetic sequence with this coding capacity or any such cloned sequence or combination thereof. For example, fusion proteins coded and
15 expressed by an entirely recombinant DNA sequence is encompassed by this invention but not to the exclusion of fusion proteins peptides obtained by other techniques.

Vectors useful for constructing eukaryotic expression systems for the production of fusion proteins comprise the fusion protein's DNA sequence, operatively linked thereto with appropriate transcriptional activation DNA sequences, such as a promoter and/or
20 operator. Other typical features may include appropriate ribosome binding sites, termination codons, enhancers, terminators, or replicon elements. These additional features can be inserted into the vector at the appropriate site or sites by conventional splicing techniques such as restriction endonuclease digestion and ligation.

Yeast expression systems, which are the preferred variety of recombinant
25 eukaryotic expression system, generally employ *Saccharomyces cerevisiae* as the species of choice for expressing recombinant proteins. Other species of the genus *Saccharomyces* are suitable for recombinant yeast expression system, and include but are not limited to *carlsbergensis*, *uvarum*, *rouxii*, *montanus*, *kluyveri*, *elongisporus*, *norbensis*, *oviformis*, and *diastaticus*. *Saccharomyces cerevisiae* and similar yeasts possess well known
30 promoters useful in the construction of expression systems active in yeast, including but not limited to GAP, GAL10, ADH2, PHO5, and alpha mating factor.

Yeast vectors useful for constructing recombinant yeast expression systems for expressing fusion proteins include, but are not limited to, shuttle vectors, cosmid plasmids, chimeric plasmids, and those having sequences derived from two micron circle plasmids. Insertion of the appropriate DNA sequence coding for fusion proteins into these vectors will, in principle, result in a useful recombinant yeast expression system for fusion proteins where the modified vector is inserted into the appropriate host cell, by transformation or other means. Recombinant mammalian expression system are another means of producing the fusion proteins for the vaccines/immunogens of this invention. In general, a host mammalian cell can be any cell that has been efficiently cloned in cell culture. However, it is apparent to those skilled in the art that mammalian expression options can be extended to include organ culture and transgenic animals. Host mammalian cells useful for the purpose of constructing a recombinant mammalian expression system include, but are not limited to, Vero cells, NIH3T3, GH3, COS, murine C127 or mouse L cells. Mammalian expression vectors can be based on virus vectors, plasmid vectors which may have SV40, BPV or other viral replicons, or vectors without a replicon for animal cells. Detailed discussions on mammalian expression vectors can be found in the treatises of Glover, *DNA Cloning: A Practical Approach*, Vols. 1-4 (Oxford, IRL Press, 1985).

Fusion proteins may possess additional and desirable structural modifications not shared with the same organically synthesized peptide, such as adenylation, carboxylation, glycosylation, hydroxylation, methylation, phosphorylation or myristylation. These added features may be chosen or preferred as the case may be, by the appropriate choice of recombinant expression system. On the other hand, fusion proteins may have its sequence extended by the principles and practice of organic synthesis.

25

Vaccines and Immunogenic Compositions

When used in vaccine or immunogenic compositions, the proteins or peptides of the present invention may be used as "subunit" vaccines or immunogens. Such vaccines or immunogens offer significant advantages over traditional vaccines in terms of safety and cost of production; however, subunit vaccines are often less immunogenic than

30

whole-virus vaccines, and it is possible that adjuvants with significant immunostimulatory capabilities may be required in order to reach their full potential.

Currently, adjuvants approved for human use in the United States include aluminum salts (alum). These adjuvants have been useful for some vaccines including hepatitis B, diphtheria, polio, rabies, and influenza. Other useful adjuvants include Complete Freund's Adjuvant (CFA), Incomplete Freund's Adjuvant (IFA), Muramyl dipeptide (MDP) (see Ellouz *et al.*, 1974), synthetic analogues of MDP (reviewed in Chedid *et al.*, 1978), N-acetylmuramyl-L-alanyl-D-isoglutamyl-L-alanine-2-[1,2-dipalmitoyl-s-glycero-3-(hydroxyphosphoryloxy)]ethylamide (MTP-PE) and compositions containing a metabolizable oil and an emulsifying agent, wherein the oil and emulsifying agent are present in the form of an oil-in-water emulsion having oil droplets substantially all of which are less than one micron in diameter (see EP 0399843).

The formulation of a vaccine or immunogenic compositions of the invention will employ an effective amount of the protein or peptide antigen. That is, there will be included an amount of antigen which, in combination with the adjuvant, will cause the subject to produce a specific and sufficient immunological response so as to impart protection to the subject from subsequent exposure to an HIV virus. When used as an immunogenic composition, the formulation will contain an amount of antigen which, in combination with the adjuvant, will cause the subject to produce specific antibodies which may be used for diagnostic or therapeutic purposes.

The vaccine compositions of the invention may be useful for the prevention or therapy of HIV-1 infection. While all animals that can be afflicted with HIV-1 can be treated in this manner, the invention, of course, is particularly directed to the preventive and therapeutic use of the vaccines of the invention in man. Often, more than one administration may be required to bring about the desired prophylactic or therapeutic effect; the exact protocol (dosage and frequency) can be established by standard clinical procedures.

The vaccine compositions are administered in any conventional manner which will introduce the vaccine into the animal, usually by injection. For oral administration the vaccine composition can be administered in a form similar to those used for the oral administration of other proteinaceous materials. As discussed above, the precise amounts

and formulations for use in either prevention or therapy can vary depending on the circumstances of the inherent purity and activity of the antigen, any additional ingredients or carriers, the method of administration and the like.

By way of non-limiting illustration, the vaccine dosages administered will typically be, with respect to the gp120 antigen, a minimum of about 0.1 mg/dose, more typically a minimum of about 1 mg/dose, and often a minimum of about 10 mg/dose. The maximum dosages are typically not as critical. Usually, however, the dosage will be no more than 500 mg/dose, often no more than 250 mg/dose. These dosages can be suspended in any appropriate pharmaceutical vehicle or carrier in sufficient volume to carry the dosage. Generally, the final volume, including carriers, adjuvants, and the like, typically will be at least 0.1 ml, more typically at least about 0.2 ml. The upper limit is governed by the practicality of the amount to be administered, generally no more than about 0.5 ml to about 1.0 ml.

Peptides of the invention corresponding to domains of the envelope protein such as V3 may be constructed or formulated into compounds or compositions comprising multimers of the same domain or multimers of different domains. For instance, peptides corresponding to the V3 domain may be circularized by oxidation of the cysteine residues to form multimers containing 1, 2, 3, 4 or more individual peptide epitopes. The circularized form may be obtained by oxidizing the cysteine residues to form disulfide bonds by standard oxidation procedures such as air oxidation.

Synthesized peptides of the invention may also be circularized in order to mimic the geometry of those portions as they occur in the envelope protein. Circularization may be facilitated by disulfide bridges between existing cysteine residues. Cysteine residues may also be included in positions on the peptide which flank the portions of the peptide which are derived from the envelope protein. Alternatively, cysteine residues within the portion of a peptide derived from the envelope protein may be deleted and/or conservatively substituted to eliminate the formation of disulfide bridges involving such residues. Other means of circularizing peptides are also well known. The peptides may be circularized by means of covalent bonds, such as amide bonds, between amino acid residues of the peptide such as those at or near the amino and carboxy termini (see U.S. Patent 4,683,136).

In an alternative format, vaccine or immunogenic compositions may be prepared as vaccine vectors which express the HIV protein or peptide of the invention in the host animal. Any available vaccine vector may be used, including live Venezuelan Equine Encephalitis virus (see U.S. Patent 5,643,576), poliovirus (see U.S. Patent 5,639,649),
5 pox virus (see U.S. Patent 5,770,211) and vaccinia virus (see U.S. Patents 4,603,112 and 5,762,938). Alternatively, naked nucleic acid encoding a protein or peptide of the invention may be administered directly to effect expression of the antigen (see U.S. Patent 5,739,118).

10 *Diagnostic Reagents*

The HIV protein or peptide compositions of the present invention may be used as diagnostic reagents in immunoassays to detect anti-HIV antibodies, particularly anti-gp120 antibodies. Many HIV immunoassay formats are available. Thus, the following discussion is only illustrative, not inclusive. See generally, however, U.S.
15 Patents 4,743,678; 4,661,445; and 4,753,873 and EP 0161150 and EP 0216191.

Immunoassay protocols may be based, for example, upon composition, direct reaction, or sandwich-type assays. Protocols may also, for example, be heterogeneous and use solid supports, or may be homogeneous and involve immune reactions in solution. Most assays involved the use of labeled antibody or polypeptide. The labels may
20 be, for example, fluorescent, chemiluminescent, radioactive, or dye molecules. Assays which amplify the signals from the probe are also known, examples of such assays are those which utilize biotin and avidin, and enzyme-labeled and mediated immunoassays, such as ELISA assays.

Typically, an immunoassay for anti-HIV antibody will involve selecting and
25 preparing the test sample, such as a biological sample, and then incubating it with an HIV protein or peptide composition of the present invention under conditions that allow antigen-antibody complexes to form. Such conditions are well known in the art. In a heterogeneous format, the protein or peptide is bound to a solid support to facilitate separation of the sample from the polypeptide after incubation. Examples of solid
30 supports that can be used are nitrocellulose, in membrane or microtiter well form, polyvinylchloride, in sheets or microtiter wells, polystyrene latex, in beads or microtiter

plates, polyvinylidene fluoride, diazotized paper, nylon membranes, activated beads, and Protein A beads. Most preferably, Dynatech, Immulon® microtiter plates or 0.25 inch polystyrene beads are used in the heterogeneous format. The solid support is typically washed after separating it from the test sample.

5 In homogeneous format, on the other hand, the test sample is incubated with the HIV protein or peptide in solution, under conditions that will precipitate any antigen-antibody complexes that are formed, as is known in the art. The precipitated complexes are then separated from the test sample, for example, by centrifugation. The complexes formed comprising anti-HIV antibody are then detected by any number of
10 techniques. Depending on the format, the complexes can be detected with labeled anti-xenogenic Ig or, if a competitive format is used, by measuring the amount of bound, labeled competing antibody. These and other formats are well known in the art.

Diagnostic probes useful in such assays of the invention include antibodies to the HIV-1 envelope protein. The antibodies to may be either monoclonal or polyclonal,
15 produced using standard techniques well known in the art (See Harlow & Lane, *Antibodies: A Laboratory Manual*, (Cold Spring Harbor, Cold Spring Harbor Laboratory Press, 1988). They can be used to detect HIV-1 envelope protein by specifically binding to the protein and subsequent detection of the antibody-protein complex by ELISA, Western blot or the like. The HIV-1 envelope protein used to elicit these antibodies can
20 be any of the variants discussed above. Antibodies are also produced from peptide sequences of HIV-1 envelope proteins using standard techniques in the art (Harlow & Lane, *supra*). Fragments of the monoclonals or the polyclonal antisera which contain the immunologically significant portion can also be prepared.

The following working examples specifically point out preferred embodiments of
25 the present invention, and are not to be construed as limiting in any way the remainder of the disclosure. Other generic configurations will be apparent to one skilled in the art. All references, including U.S. or foreign patents, referred to in this application are herein incorporated by reference in their entirety.

30 Examples

The following methods were used in the Examples:

Reference Serum Donor Envelope Gene Cloning

The donor of the HIV-1 Neutralizing Serum (2) (Reference 2), available in the NIH AIDS Research and Reference Reagent Program (Catalog Number: 1983) is a participant in a long term cohort study at the Clinical Center, NIH (Vujcic *et al.*, 1995).

5 The blood used to prepare Reference 2 had been collected in the Spring of 1989.

Peripheral blood mononuclear cells that had been cryopreserved from donations obtained approximately six months and one year prior to the time of Reference 2 collections were used as sources of DNA for *env* gene cloning. The cells had not been stored to maintain viability. DNA was extracted using phenol/chloroform from approximately $1-3 \times 10^6$

10 cells from each donation (Quinnan *et al.*, 1998). The DNA was used as template in a nested polymerase chain reaction, similar to that described previously, except rTth was used as the DNA polymerase, following the manufacturer's instructions (Barnes, 1992; Cariello *et al.*, 1991). The DNA was cloned into the expression vector pSV7d, as previously described (Quinnan *et al.*, 1998; Stuve *et al.*, 1987).

15

Other env Gene Clones and Virus Pools

The following HIV-1 *env* clones in the expression vector pSV3 were obtained from the AIDS Research and Reference Reagent Program, 93MW965.26 (clade C), 92RWO20.5 (clade A), 93TH966.8 (clade E), 92UG975.10 (clade G) (Gao *et al.*, 1994).

20 The production of *env* clones from the molecular virus clones NL43, AD8, and SF162 has been previously described (Quinnan *et al.*, 1998; Adachi *et al.*, 1986; Theodore *et al.*, 1996; Englund *et al.*, 1995). *env* gene of the Z2Z6 strain was cloned similarly, using molecular virus clone plasmid as template in polymerase chain reaction, and cloning the genes into the plasmid pSV7d (Seth *et al.*, 1993). The production of primary isolate *env*

25 clones from participants in the Multicenter AIDS Cohort Study, designated here P9 and P10, has also been previously described (Quinnan *et al.*, 1998). P9 and P10- virus pools were prepared by single subpassages of the cell culture media from primary cultures in PHA blasts (Quinnan *et al.*, 1998). The use of molecular virus clones for preparation of virus pools of NL43 in H9 cells, and NL(SF162) and AD8, in PHA blasts, has also been

30 previously described (Quinnan *et al.*, 1998).

Cell Cultures

The H9 cell line was obtained from Robert Gallo (Mann *et al.*, 1989). The Molt 3 cell line was obtained from the American Type Culture Collection, Rockville, MD (ATCC). (Daniel *et al.*, 1988) The HOS cell lines expressing CD4 and various
5 coreceptors for HIV-1 were obtained from the NIH AIDS Research and Reference Reagent Program, as was the PM1 cell line (Deng *et al.*, 1996; Landau *et al.*, 1992; Lusso *et al.*, 1995). The 293T cell line was obtained from the ATCC, with permission from the Rockefeller Institute (Liou *et al.*, 1994). The H9, Molt3 and PM1 cell cultures were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum and
10 antibiotics (Gibco). The HOS and 293T cells were maintained in Dulbecco's Minimal Essential Medium (Gibco), with similar supplements, except that the HOS cell medium was supplemented with puromycin for maintenance of plasmid stability. Cryopreserved human peripheral blood lymphocytes were stimulated with PHA and used for virus infections (Quinnan *et al.*, 1998; Mascola *et al.*, 1994).

15

Reverse Transcriptase Assay

Reverse transcriptase activity was assayed as previously described (Park *et al.*, 1998).

20 *Virus Neutralization Assays*

The virus NL43 was used in neutralization assays which employed Molt3 cells as target cells and used giant cell formation for endpoint determination, as previously described (Vujcic *et al.*, 1995). The amounts of virus used were sufficient to result in the formation of 30-50 giant cells per well (Vujcic *et al.*, 1995; Lennette, 1964). The viruses,
25 NL(SF162) and AD8, P9 and P10 were tested for neutralization in PHA stimulated human lymphoblasts in the presence of IL-2 (Quinnan *et al.*, 1998; Mascola *et al.*, 1994). In the latter assays ten percent of the cell suspension was removed each week, fifty percent of the medium was changed each week, and medium was sampled twice weekly from each well for reverse transcriptase assay. The reverse transcriptase assays were
30 performed on the test samples from the first sampling date at which the non-neutralized control wells had reverse transcriptase activity about 10-20 x background, generally on

day 14 or 17 of the assay. The neutralization endpoint was considered to be the highest dilution of serum at which reverse transcriptase activity was reduced at least fifty percent. The Reference Neutralizing Sera 1 and 2 and the Negative Reference Serum were used as positive and negative controls (NIH AIDS Research and Reference Reagent Program)

5

Pseudovirus Construction and Assays of Pseudoviruses for Infectivity and Neutralization

Pseudoviruses were constructed and assayed using methods similar to those described previously (Quinnan *et al.*, 1998; Deng *et al.*, 1996; Park *et al.*, 1998). pSV7d-*env* plasmid DNA and pNL43.luc+.E-R- were cotransfected into 70 to 80% confluent
10 293T cell cultures using the calcium phosphate/Hepes buffer technique, following manufacturer's instructions (Promega, Madison, WI), in 24 well plastic tissue culture trays or 25 cm² flasks (Quinnan *et al.*, 1998; Deng *et al.*, 1996; Park *et al.*, 1998). After 24 hours the medium was replaced with medium containing one mM sodium butyrate (Quinnan *et al.*, 1998; Park *et al.*, 1998). Two days after transfection medium was
15 harvested, passed through a 45µm sterile filter (Millipore Corp, Bedford, MA), supplemented with an additional 20% fetal bovine serum and stored at -80°C.

Pseudovirus infectivity was assayed in PM1 or HOS-CD4 cells expressing various co-receptors. Transfection supernatants were serially diluted and inoculated into cells in 96 well plates, 50 µl per well. Assays were routinely performed in triplicate. The
20 cultures were incubated for four days, centrifuged at 400 x g for ten minutes if PM1 cells were used, and medium removed by aspiration. The cells were washed twice with phosphate buffered saline, lysed with 25 µl cell culture lysing reagent according to the manufacturer's instructions (Promega, Madison, WI); the cells were then tritiated into the medium, and 10 µl of the suspensions were transferred to wells of 96 well luminometer
25 plates. Substrate was added in 100 µl volumes automatically, and the luminescence read using a MicroLumatPlus luminometer (EG&G Berthold, Hercules, CA). Mock PV controls were used in each assay consisting of media harvested from 293T cell cultures transfected with pSV7d (without an *env* insert) and pNL43.Luc.E-R-, and processed in the same way as cultures for PV preparation. Infectivity endpoints were determined by a
30 modified Reed Munch method; an individual well was considered positive if the luminescence was at least 10-fold greater than the mock control, and the endpoint was

considered to be the highest dilution at which the calculated frequency of positivity was \geq 50% (Quinnan *et al.*, 1998; Park *et al.*, 1998; Lennette, 1964). Luminescence resulting from infection with minimally diluted samples was generally about 10,000-fold greater than background.

5 Neutralization tests were performed using PM1 or HOS-CD4 cells. Aliquots of 25 μ l of two-fold serial serum dilutions were mixed with equal volumes of diluted PV in wells of 96 well plates. The PV dilutions were selected so as to expect luminescence in the presence of non-neutralizing serum of about 100-fold of background. Assays were performed in triplicate. The virus serum mixtures were incubated for sixty minutes at
10 40°C , after which 150 μ l aliquots of PM1 cell suspensions were added, which each contained 1.5×10^4 cells, or the suspensions were transferred to wells containing HOS-CD4 cells. The assays were then processed similarly to the infectivity assays. The neutralization endpoints were calculated by a modified Reed-Munch method in which the endpoint was considered to be the highest serum dilution calculated to have a frequency
15 of \geq 50% for reducing luminescence by \geq 90% compared to the non-neutralized control. PV titrations were conducted in duplicate in parallel with each neutralization assay.

Nucleic Acid Sequencing

Nucleotide sequence analysis was performed using the di-deoxy cycle sequencing
20 technique and AmpliTaq FS DNA polymerase, according to manufacturer's directions (Perkin Elmer Applied Biosystems, Foster City, CA). After the sequencing reaction the DNA was purified using Centriflex Gel Filtration Cartridges (Advanced Genetic Technologies, Gaithersburg, MD). Sequencing gels were run and analyzed using an Applied Biosystems Prism, Model 377 DNA Sequencer. Sequencing was performed on
25 both strands. Sequence alignment was performed using the Editseq SEQMAN, and Megalign programs in DNA Star according to the method of Higgins and Sharp (1989).

Example 1: Comparability of Clones Isolated from Different Time Points

From the samples of patient cells from each of the two time points, *env* clones
30 were recovered which encoded proteins which were capable of mediating pseudovirus entry into target cells. Two such clones from each time point were further characterized.

As shown in Table 1, the envelopes of all four clones mediated infection for PM1 cells and were neutralized comparably by References 1 and 2. Pseudoviruses carrying envelopes corresponding to each clone were also tested for infectivity for HOS-CD4 cells expressing either CXCR4 or CCR5, and all four were infectious only for the cells
5 expressing CCR5, as shown in Table 2. Nucleotide sequences including the V3 regions were analyzed for each clone, with more than 300 bases assigned for each, and no differences between the clones were found (results not shown). Based on the absence of demonstration of differences in these assays, a single clone from the March sample was selected for use in subsequent assays, and is designated R2, hereafter.

10

Example 2: Clone R2 Genotype and Host Range Phenotype

The complete nucleotide sequence of the *env* gene clone R2 was determined, and found to have an open reading frame of 2598 bases (Genbank Accession Number: AF128126) The amino acid sequence deduced from this sequence is shown in Table 3
15 (SEQ ID NO:1). There are thirty predicted glycosylation sites, compared to twenty-nine in the consensus clade B sequence; four consensus glycosylation sites are lacking in R2, including those at residues 146, 215, 270, and 368 (numbering according to the Human Retroviruses and AIDS Database clade B consensus sequence), in the V2, C2, C2 and V4 regions of gp120, respectively (Myers *et al.*, 1993). The consensus glycosylation
20 sequences at residues 215 and 270 are highly and moderately variable, respectively.

Genotypic analyses conducted included evaluation of the gp120 and gp41 nucleotide coding sequences in comparison to those of a number of strains of clades A through G, as shown in Figure 1 (Saitou *et al.*, 1987; Myers & Miller, 1988). Both coding regions were more closely related to clade B than non-clade B sequences.

25 Comparative analyses of regions of the predicted gp120 and gp41 amino acid sequences were also performed (results not shown). The regions analyzed included: each constant and variable region of gp120; the proximal gp41 ectodomain including the leucine zipper region; the part of gp41 extending from the end of the leucine zipper to the second cysteine; the remaining gp41 ectodomain, and the transmembrane region; and the
30 cytoplasmic region. R2 consistently related more closely with the clade B sequences than the others.

Example 3: Comparative Sensitivity of R2 and Other Clade B Viruses and Pseudoviruses to Neutralization by Sera from Individuals with Clade B Infections

The neutralization of R2 pseudovirus was compared to other clade B viruses and
 5 pseudoviruses as shown in Figure 2. Of the five virus-pseudovirus comparisons made
 (P9, P10, NL43, AD8 and SF162 V and PV), there were no significant differences in the
 neutralization of matched viruses and pseudoviruses by paired t test (statistical results not
 shown). Each of the pseudovirus preparations was neutralized by seven, eight, or nine of
 the sera tested, and the geometric mean titers ranged from 1:13.9 to 1:56, while the R2-
 10 PV was neutralized by all ten of the sera tested, with a geometric mean titer of 1:73.5.
 Although the neutralization titers of each of the different sera against R2 and the other
 pseudoviruses were frequently within four-fold, the neutralization of R2-PV was
 significantly greater by paired t test than four of the other PV preparations.

15 *Example 4: Comparative neutralization of pseudoviruses expressing R2 and other envelopes of diverse subtypes by sera from diverse subtype infections.*

The results of comparative neutralization testing using sera from individuals
 infected with HIV- I strains of subtypes A, C and E, and the Reference 1 and 2, and one
 Thai clade B serum are shown in Table 4. Reference 2 neutralized the pseudovirus
 20 expressing the homologous R2 envelope at the modest titer of 1:64 in the experiment
 shown and within two-fold of this titer in many other experiments. It neutralized the
 other seven pseudoviruses tested at low to moderate titers, as well. The R2 pseudovirus
 was neutralized by seventeen of twenty-four sera, including sera from people infected
 with each of the clades A-F. The other two clade B pseudoviruses were neutralized less
 25 frequently and were also neutralized infrequently by the clade E sera. The frequency of
 neutralization by sera from individuals infected with different clades was not significantly
 skewed for any of the other four pseudoviruses. Clade A, C, D and G pseudoviruses were
 neutralized by eight, seventeen, six and three of the seventeen sera tested, respectively.
 The clade C pseudovirus was substantially more sensitive to neutralization, in general
 30 than the others tested. The clade E pseudovirus was neutralized by five of five clade D

sera and seven of eight clade E sera but only one of the sera from people infected by other clades.

Example 5: Synthetic peptides generated from V3 amino acid sequences from R2 strain.

5 R2 strain V3 peptides were synthesized using an automated ABI synthesizer and Fmoc chemistry (Zeng *et al.*, 1997). The sequences of these peptides were KSIPMGPGRAFYTGGQI (SEQ ID NO:2) and CSRPNNNTRKSIPMGPGRAFYTGGQIIGDIRQAHC (SEQ ID NO:3). The mutant R2(313-4PM/HL, 325Q/D) V3 peptide was prepared similarly. Strain 93TH966.8 V3
10 peptide, sequence: CTRPSNNTRTSTTIGPGQVFYRTGDITGNIRKAYC (SEQ ID NO:4) was synthesized using the same methods. The peptides were purified using C18, acetonitrile-in-water gradient chromatography with a Waters High Performance Liquid Chromatograph. Sequences of the purified peptides were verified using an ABI automated sequencer. Peptides were lyophilized and stored at 4-8°C. Preparation of a
15 linear MN strain V3 peptide has been described previously (Carrow *et al.*, 1991). Cyclic MN strain 35-mer peptide was obtained from the AIDS Research and Reference Reagent Program (Catalog #1841) provided by Catasti *et al.*, (1996).

The R2 V3 35-mer was insoluble in water, while all other peptides tested were soluble in water to at least 10 mg/ml. To obtain cyclic peptides, solutions of the R2 and
20 R2(313-4PM/HL, 325Q/D) V3 35-mers in dimethylsulfoxide (DMSO), 10 mg/ml, were diluted 1:10 in water at room temperature or 37°C and the pH was adjusted to 8.5 with ammonium hydroxide. These solutions were aerated by bubbling air through the solutions for periods ≥ 1 hour. Following aeration, the pH was adjusted to 7.4 using hydrochloric acid. A portion of the R2 35-mer peptide precipitated during these
25 procedures. To obtain an approximate quantitation of the amount of R2 V3 35-mer that remained in solution, the turbidity of the suspension was determined at 480 nm wavelength visible light using a spectrophotometer. The spectrophotometer was blanked with a solution of 10 percent DMSO in water, and a standard curve was produced using slurries of known amounts of the 35-mer peptide suspended in water. The amount of
30 precipitate estimated by turbidity was subtracted from the amount of peptide added at the beginning of the preparation procedure to estimate the amount remaining in solution. The

solubility of the oxidized R2 35-mer peptide in 10 percent DMSO solution at pH=7.4 was estimated to be 300-350 $\mu\text{g/ml}$ when processed at room temperature, or 850-900 $\mu\text{g/ml}$ when processed at 37°C. Peptides were sterilized by passage through 0.22 μ pore size filters prior to use.

5

Example 6: Peptide blocking of neutralizing antibody activity against clone R2 pseudovirus.

The neutralization blocking effects of synthetic V3 peptides were examined to test the contribution of V3-anti-V3 interactions in the neutralizing cross reactivities of Reference 2 and clone R2. The blocking effects of peptides on neutralizing activity of Reference 2 against clone R2 pseudovirus are shown in Figure 3A. Usually, the linear 17-mer peptide had no inhibitory effect on neutralization, as shown. In only one of several experiments two-fold reduction of neutralization was observed in the presence of 17-mer peptide. Concentration-dependent inhibitory effects of the cyclic 35-mer R2 V3 peptide on neutralization of clone R2 pseudovirus by Reference 2 was observed in the experiment shown and in numerous other similar experiments. Maximum effect was observed at approximately 15 $\mu\text{g/ml}$. No inhibitory effect was observed using a cyclic peptide homologous to the V3 region of the HIV-1 93TH966.8 strain.

The comparative effects of the R2 and MN strain V3 peptides on neutralization of the R2 and MN strain pseudoviruses are shown in Figure 3B. The results shown are representative of two additional experiments. Only the cyclic R2 V3 peptide produced consistent blocking of R2 pseudovirus neutralization. The linear R2 and MN, and the cyclic MN peptides did not block R2 neutralization in two experiments and blocked only two-fold in a third experiment. In contrast, the MN cyclic and linear peptides consistently inhibited MN strain neutralization eight- to sixteen-fold in these experiments, and the R2 peptides had consistent two-fold inhibitory effects on neutralization of the MN strain. These effects of MN peptides on MN strain neutralization are consistent with previous reports (Carrow *et al.*, 1991; Park *et al.*, 1999).

Example 7: Cyclic R2 V3 peptide inhibition of neutralization of R2 pseudoviruses by sera from MACS patients.

Inhibition of heterologous serum neutralization of R2 pseudovirus by cyclic R2 V3 peptide was evaluated to determine if cross reactivity of these sera with R2 included effects of anti-V3 antibodies. The comparative neutralization titers of sera from ten patients from the MACS against clone R2 pseudovirus in the presence and absence of cyclic R2 V3 peptide are shown in Figure 4A (Quinnan *et al.*, 1998). These sera have been described previously, and have been shown to neutralize primary HIV-1 enveloped pseudoviruses cross reactively, but to a lesser extent than Reference 2 (Zhang *et al.*, 1999). Each serum was tested twice. Seven of the sera appeared to be inhibited at least two-fold in one or both experiments. The geometric mean inhibitory effect of all the tests was 1.9-fold. The results of twelve tests conducted at the same times as those tests shown in Figures 4A and 4B are shown for Reference 2; the geometric mean inhibitory effect was 3.56.

Example 8: Cyclic R2 V3 peptide inhibition of Reference 2 neutralization of pseudoviruses expressing envelopes from the MACS patients.

Inhibition of Reference 2 neutralization of pseudoviruses expressing heterologous envelopes by cyclic R2 V3 peptide was evaluated to determine whether anti-V3 antibody contributed to the neutralizing cross reactivity of Reference 2. The results of these experiments are shown in Figure 4B. Each pseudovirus was tested two or three times. The peptide appeared to exert a two-fold inhibitory effect in one, two, or three of the experiments using each of the six pseudoviruses. The geometric mean inhibitory effect was 1.6-fold.

Example 9: Induction of cross-reactive neutralizing antibodies in mice following immunization with recombinant delivery vectors encoding HIV-1 envelope proteins.

The DNA clone encoding the R2 envelope was introduced into an expression vector which can be used to express the envelope protein complex *in vivo* for immunization. The recombinant delivery vector expressing the R2 envelope clone was been administered to mice, both in its full length, encoding both gp120 and gp41, or in a truncated form. The truncated form is secreted by cells which express gp140. Both the full-length and truncated form of these constructs induced neutralizing antibodies in mice.

The mice which received the gp140 construct, which includes the V3 region, have developed neutralizing antibodies which neutralize at least three different strains of HIV-1, including the R2 strain, a macrophage tropic laboratory strain known as SF162, and a primary strain which is not laboratory adapted. The amount of cross-reactivity
5 observed exceeds that induced by most or all other HIV immunogens that have been tested as single agents.

Table 1. Comparative Neutralization of Pseudoviruses Expressing Multiple Envelope Clones From Donor 2

Serum	Neutralization Titer Against Clone			
	10.1	10.2	3.1	3.2
Reference 1	1:32	1:64	1:32	1:64
Reference 2	1:128	1:128	1:128	1:128

Table 2. Coreceptor Dependency of R2 Pseudovirus Entry Into HOS-CD4 Cells

Pseudo-virus	Infectivity Titer						In PM1 Cells
	In HOS-CD4 Cells Expressing						
	CCR1	CCR2b	CCR3	CCR4	CCR5	CXCR4	
R2	<1:4	<1:4	<1:4	<1:4	1:64	<1:4	1:32
P9	<1:4	<1:4	<1:4	<1:4	1:256	<1:4	1:8
NL4-3	<1:4	<1:4	<1:4	<1:4	1:32	>1:256	1:8
AD8	<1:4	<1:4	<1:4	<1:4	1:256	<1:4	1:32

Table 3. Inferred Amino Acid Sequence of the R2 Envelope Clone from Donor 2.

Amino Acid Residue ^a	Residue
	Number
MRVKGIRRRNY QHWWGWGTML LGLLMICSAT EKLWVTVYYG VPVWKEATTT	50
LFCASDAKAY DTEAHNVWAT HACVPTDPNP QEVELVNVTE NFNMWKNNMV	100
EQMHEDIISL WDQSLKPCVK LTPLCVTLNC TDLRNTTNTN N STDNNNSNS	150
EGTIKGGEMK NCSFNIATSI GDKMQKEYAL LYKLDIEPID NDNTSYRLIS	200
CNTSVITQAC PKISFEPIPI HYCAPAGFAI LKCNDKKFSG KG SCK NVSTV	250
QCTHGIRPVV STQLLLNGSL AEEEVVIRSE N FTNNAKTII VQLREPVKIN	300
CSRPNNNTRK SIPMGPGRAF YTTGQIIIGDI RQAH C NISK T N WTNALKQVV	350
EKLGEQ F NKT KIVFTNSSGG DPEIVTHSFN CAGEFFYC N T TQLFDSIWNS	400
ENGTWNITRG LNNTGRNDTI TLPCRIKQII NRWQEVGKAM YAPPIKGNIS	450
CSSNITGLLL TRDGGKDDNS RDGNETFRPG GGDMRDNWRS ELYKYKVVKI	500
EPLGVAPT K A KRRVVQREER AVGLGAMFIG FLGAAGSTM G AASVTLTVQA	550
RQLLSGIVQQ QSNLLRAIEA QOHLQLTLVW GIKQLQARIL AVERYLK D QQ	600
LLGIWGCSGK LICTTTVP W N ASWSK N K T LE AIWNNMTWMQ WDKEIDNYTK	650
LIYSLIEESQ IQQEKNEQEL LELDKWANLW NWFDISNWLW YIKIFIMIVG	700
GLVGLRIVFV VLSIVNRVRQ GYSPLSFQTR LPAPRGPD R P EEIEEEGGDR	750
DRDRSGLLVD GFLT L IWVDL RSLCLFSYHR LRDLL L LIVTR IVELLGRRGW	800
EILKYWWNLL QYWSQELKNS AVSLFNATAI AVAEGTDRVI EVLQ R VGRAL	850
LHIPTRIRQ G LERALL	866

^aAmino acid residues are identified by standard single letter designations. Predicted N-linked glycosylation sites are indicated by shading and bolding.

Table 4. Neutralization of Pseudoviruses Expressing Envelopes of Various Clades by Sera from People Infected with Various Clades of HIV-1

Clade	Serum ^b	NA Titer Against Pseudovirus (Clade) ^a							
		R2 (B)	P9 (B)	P10 (B)	BR020 (A)	MW965 (C)	Z2Z6 (D)	TH966 (E)	UG975 (G)
B	Ref 1	32	16	32	<10	256	10	<8	<10
	Ref 2	64	32	64	10	128	40	8	10
	WR8465	20	NT ^c	80	<10	640	10	<10	10
A	37570	320	160	20	80	2560	<10	<10	<10
	35374	40	<10	<10	<10	640	<10	<10	<10
	35837	40	20	<10	80	2560	<10	<10	<10
C	5107	40	10	<10	10	1280	<10	<10	<10
	5708	10	<10	<10	<10	320	<10	<10	<10
	5218	80	<10	<10	<10	1280	<10	<10	<10
D	UG9240	<10	NT	NT	NT	NT	NT	20	NT
	UG9370	<10	NT	NT	NT	NT	NT	10	NT
	UG9386	<10	NT	NT	NT	NT	NT	10	NT
	UG93097	10	NT	NT	NT	NT	NT	10	NT
	UG94118	10	NT	NT	NT	NT	NT	20	NT
E	WR5659	10	<10	<10	<10	20	<10	40	<10
	WR5901	<10	<10	<10	40	320	10	40	10
	WR8177	<10	<10	<10	40	640	10	80	<10
	WR8657	<10	<10	10	10	640	<10	80	<10
	WR8593	<10	<10	<10	<10	160	10	40	<10
	1008	<10	<10	<10	<10	10	<10	<10	<10
	1053	20	<10	<10	<10	40	<10	20	<10
	1062	20	10	<10	10	320	<10	20	<10

F	BR9318	<10	NT	NT	NT	NT	NT	<10	NT
	BR93019	10	NT	NT	NT	NT	NT	<10	NT
	BR93020	20	NT	NT	NT	NT	NT	<10	NT
	BR93029	10	NT	NT	NT	NT	NT	<10	NT

^aNeutralization titers are the dilutions at which 90% inhibition of luminescence was observed.

^bSera were the Reference Neutralizing Human Serum 1 and 2, or were provided by Dr. J. Mascola, HIVNET, or the UNAIDS Program, as described in the text.

^cNT=not tested.

Table 5. Comparison of V3 Region Amino Acid Sequences of Clone R2 with Phenetic Subgroup Consensus Sequences 1 Through 13 and Clade A Through E Consensus Sequences.^a

Clone, Subgroup or Clade	V3 Region Amino Acid Sequence
R2	NNTR.KSIPMGPGRAFYTTGQIIIGDIRQAHC
PHENETIC 1	-----HI-----D-----
PHENETIC 2	-----SI-----A--E-----
PHENETIC 3	-----SI-----A--K-----
PHENETIC 4	-----RI---Q---A--D-----
PHENETIC 5	-----HI-----A--K-----
PHENETIC 6	K--RRR-H.I-----K-----
PHENETIC 7	----.T--TI---QV--R--K-----
PHENETIC 8	KKM-.T-ARI---V-HK--D---S-TK-Y-
PHENETIC 9	----.Q-THI---Q-L---.D---K-----
PHENETIC 10	----.QGTHI-----Y---.N-----
PHENETIC 11	----.QRTSI-Q-QAL---.E-R-----A-
PHENETIC 12	D-IKIQRT-I-Q-Q-L---RITGYI.G----
PHENETIC 13	Q-K-.QGT-I-L-Q-L---R.-K---K---
CLADE A	----.---VHI---Q---A--D-----
CLADE B	----.---HI-----E-----
CLADE C	----.---RI---QT-YA--D-----
CLADE D	----.QRTHI---Q-L---.R-----
CLADE E	----.T--TI---QV--R--D-----K-Y-

^aDashes indicate residues at which the individual sequences are identical to R2. The periods indicate sites of insertions or deletions.

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What is claimed:

1. An isolated HIV envelope protein or fragment thereof which, when
administered to a mammal, induces the production of broadly cross-reactive neutralizing
5 anti-serum against multiple strains of HIV-1.
2. An isolated HIV envelope protein comprising the amino acid sequence of SEQ
ID NO:1.
- 10 3. An isolated HIV envelope protein or fragment thereof comprising a proline at a
position corresponding to amino acid residue 313, a methionine at a position
corresponding to amino acid residue 314 and a glutamine at a position corresponding to
amino acid residue 325 of SEQ ID NO:1.
- 15 4. An isolated HIV envelope protein or fragment thereof comprising a V3 region
having the amino acid sequence P M X₁ X₂ X₃ X₄ X₅ X₆ X₇ X₈ X₉ X₁₀ Q, wherein X₁-X₁₀
are a natural or non-natural amino acid.
- 20 5. A vaccine composition comprising an isolated HIV-1 envelope protein or
fragment thereof of any one of claims 1-4 and a pharmaceutically acceptable carrier.
6. An immunogenic composition comprising an isolated HIV-1 envelope protein
or fragment thereof of any one of claims 1-4 and a pharmaceutically acceptable carrier.
- 25 7. An isolated nucleic acid molecule encoding the HIV-1 envelope protein or
fragment thereof of any of claims 1-4.
8. A fusion protein comprising all or a portion of a microbiological antigen into
which any one of the proteins of claims 1-4 has been inserted.

30

9. A recombinant delivery vector encoding a fusion protein comprising all or a portion of a microbiological antigen into which any one of the proteins of claims 1-4 has been inserted.

5 10. A vaccine composition comprising any one of the recombinant delivery vectors of claim 9 and a pharmaceutically acceptable carrier.

11. An immunogenic composition comprising any one of the recombinant delivery vectors of claim 9 and a pharmaceutically acceptable carrier.

10

12. A recombinant delivery vector encoding an attenuated virus further comprising a nucleotide sequence encoding one or more of the proteins of any one of claims 1-4.

15 13. The recombinant delivery vector of claim 12 wherein the attenuated virus is selected from the group comprising HIV, encephalitis virus, poliovirus, poxvirus and vaccinia virus.

14. A vaccine composition comprising any one of the recombinant delivery
20 vectors of claim 12 and a pharmaceutically acceptable carrier.

15. An immunogenic composition comprising any one of the recombinant delivery vectors of claim 12 and a pharmaceutically acceptable carrier.

25 16. A method of generating antibodies in a mammal comprising administering one or more of the proteins or fragments thereof of any one of claims 1-4, in an amount sufficient to induce the production of the antibodies.

17. A method of generating antibodies in a mammal comprising administering a
30 DNA or mRNA sequence encoding any one of the proteins or fragments thereof of claims 1-4, in an amount sufficient to induce the production of the antibodies.

18. The method of claim 17, wherein said DNA is naked DNA.

19. A diagnostic reagent comprising one or more of the isolated HIV-1 envelope proteins or fragments thereof of any one of claims 1-4.

5

20. A method of detecting HIV-1 antibodies in a sample comprising the step of determining whether antibodies in the sample bind to one or more of the HIV-1 envelope proteins or fragments thereof of claims 1-4.

10

21. A cyclic peptide comprising the amino acid sequence of either claims 3 or 4.

22. An isolated antibody which specifically recognizes the protein of claims 3 or 4.

15

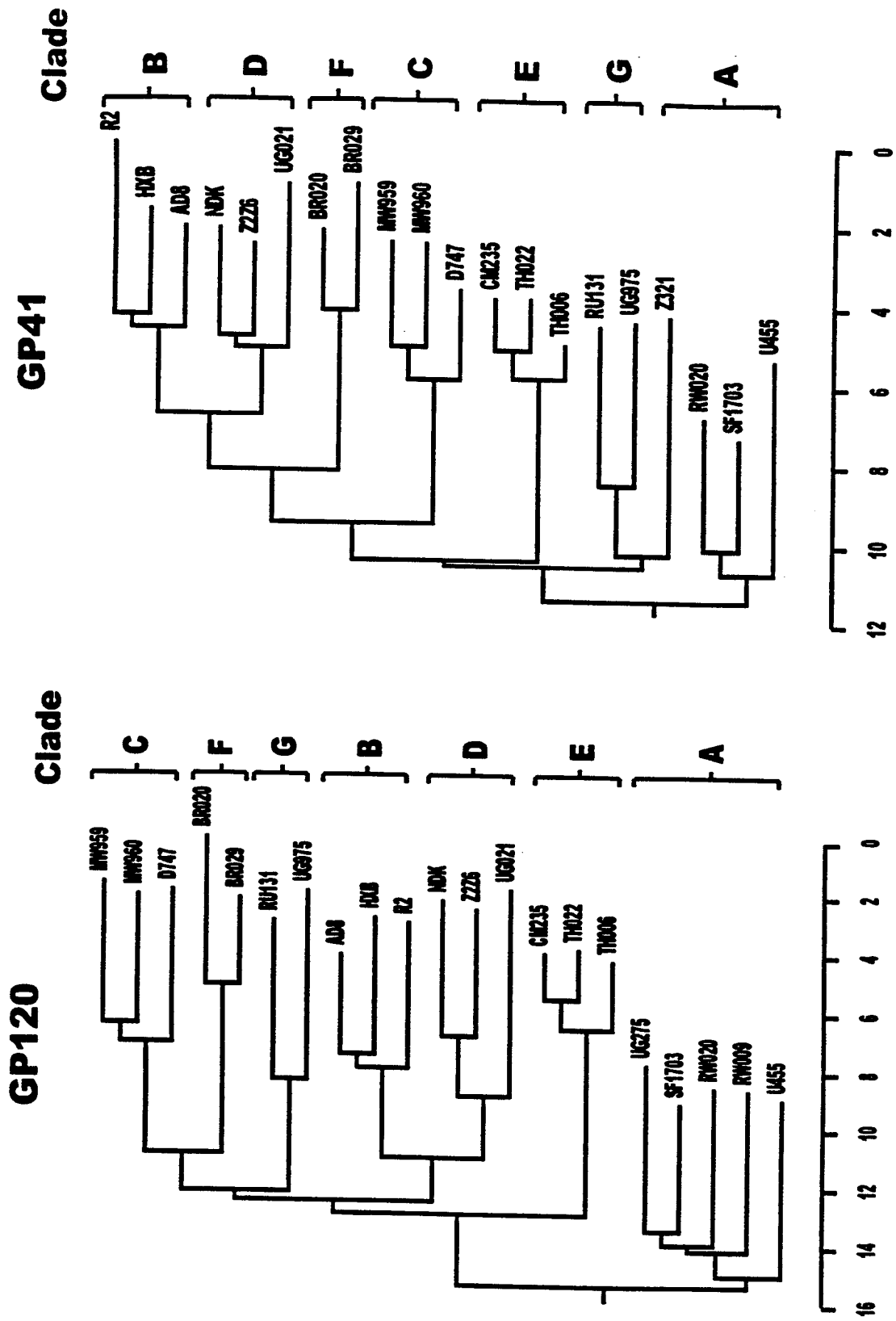


FIG. 1

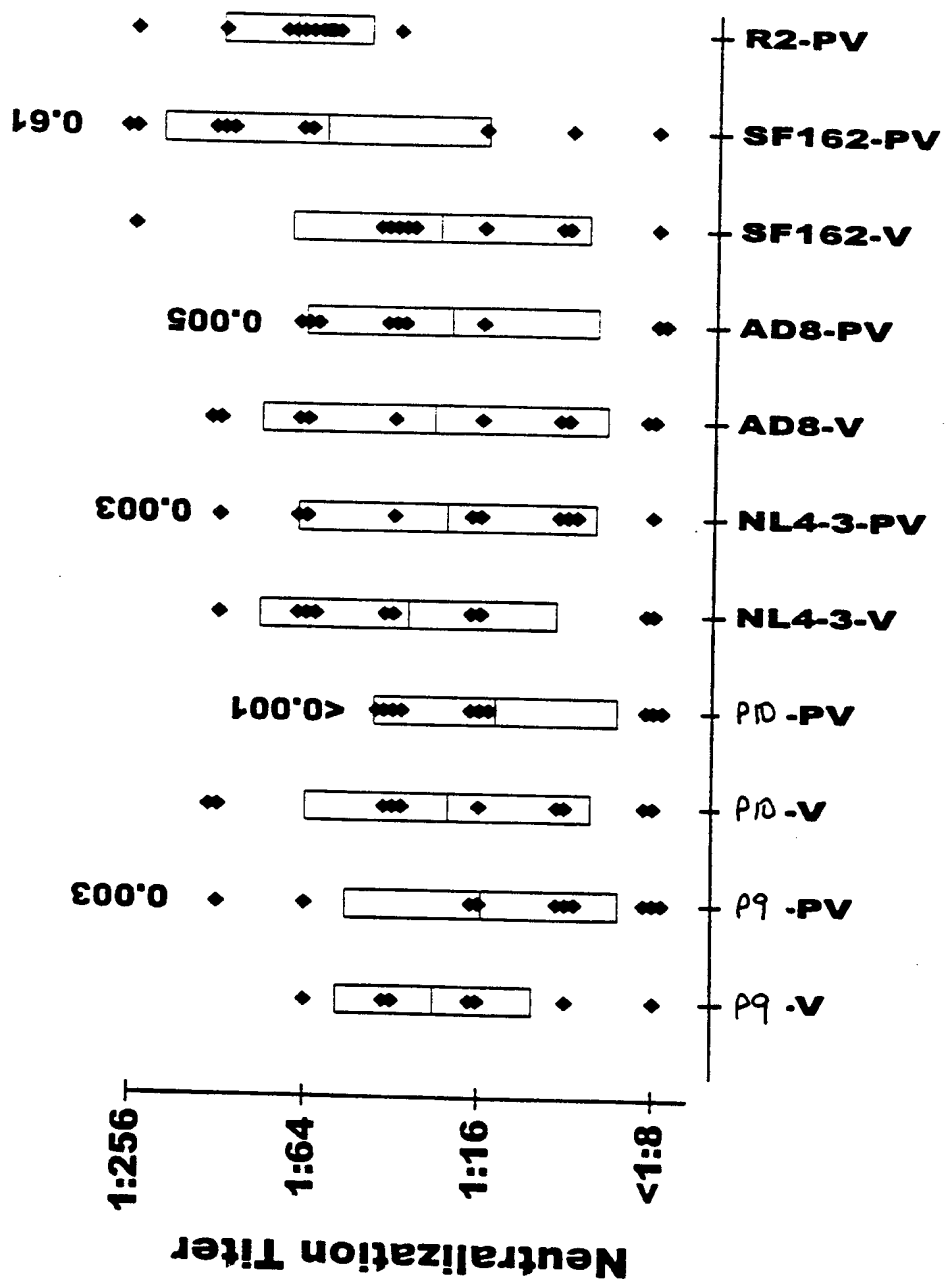
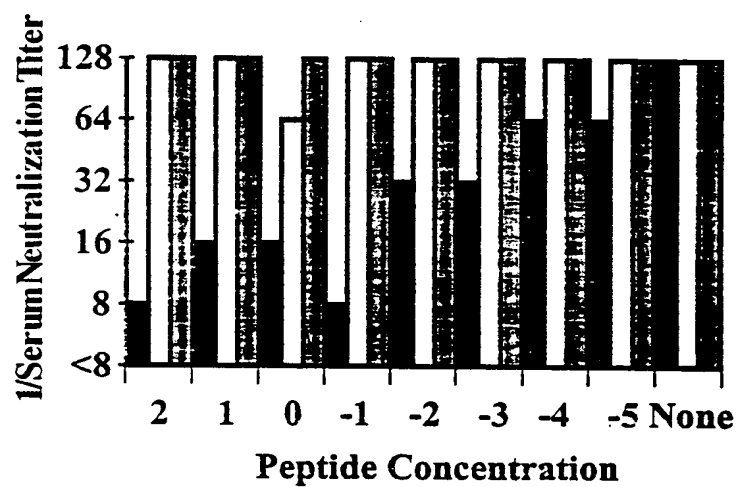
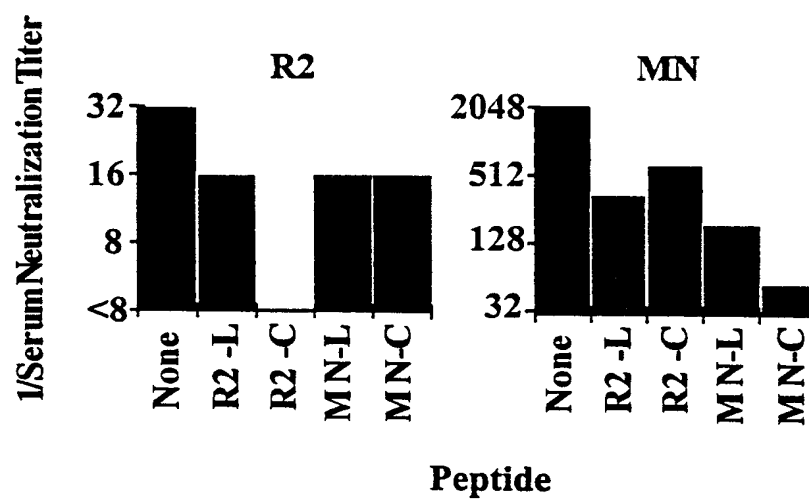
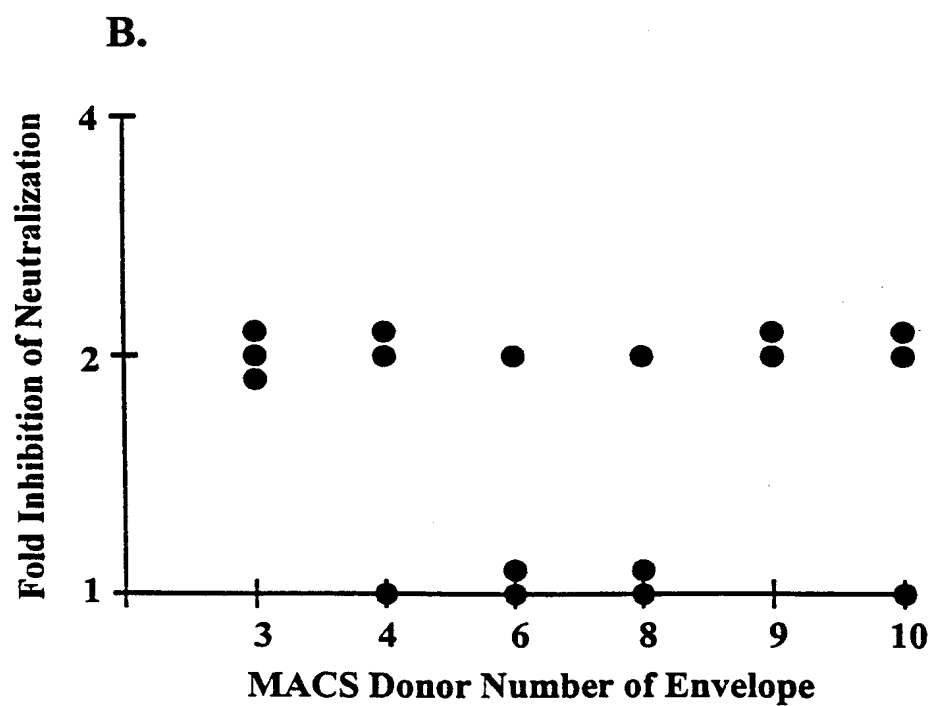
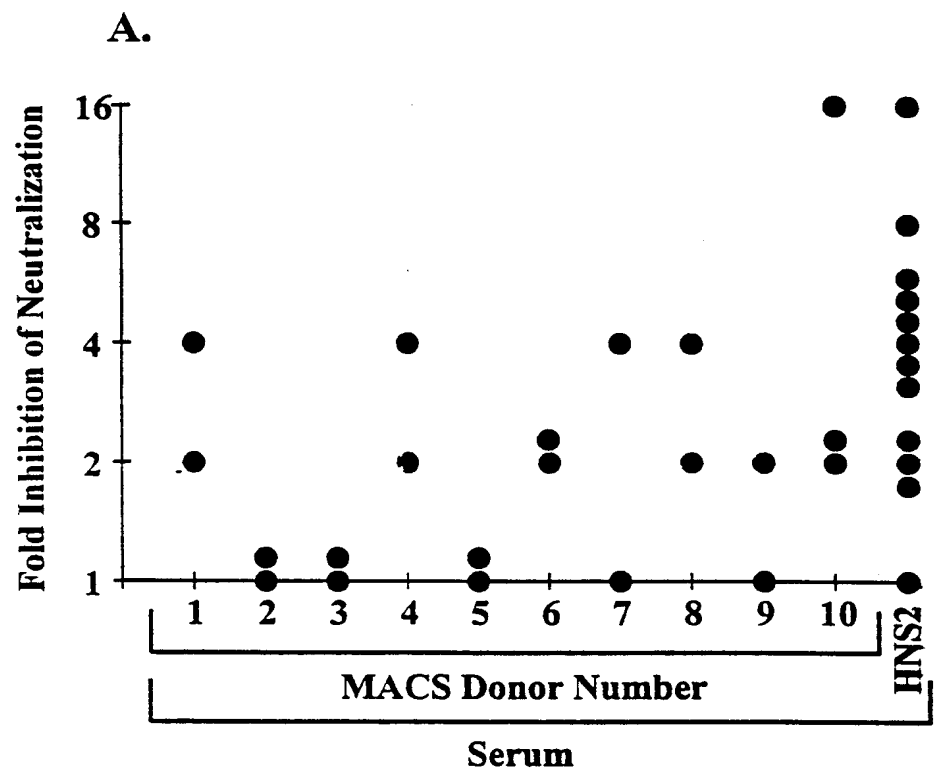


FIG. 2

A**B****FIG. 3**

**FIG. 4**

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/17596

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A61K 48/00, 38/00; C12P 21/00; C07K 5/00

US CL : 424/93.1; 514/44, 2-21; 435/71.1; 530/300

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/93.1; 514/44, 2-21; 435/71.1; 530/300

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WEST 1.2, STN, MEDLINE, terms: HIV-1 envelope, neutralizing antibody, multiple strains.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,622,933 A (SABATIER et al) 22 April 1997, especially Abstract.	1, 5-6, 16, 19-20
X	US 5,756,674 A (KATINGER et al) 26 May 1998, especially Abstract and column 3, lines 20-24.	1, 5-20
X	US 5,439,809 A (HAYNES et al) 08 August 1995, especially Abstract and columns 7 and 8.	1, 5-20

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Z* document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

10 OCTOBER 1999

Date of mailing of the international search report

21 OCT 1999

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/17596

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 2-4 AND 21-22
because they relate to subject matter not required to be searched by this Authority, namely:

The claims recite peptide sequence of SEQ ID NO:1 and depend therefrom which cannot be searched other than by a sequence search. However, no CRF for this case has been filed. Therefore, the claims are unsearchable.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.